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RAPID COMMUNICATION

Absence of Coreceptor Switch with Disease Progression in Human Immunodeficiency Virus Infections in India

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The envelope glycoprotein of the human immunodeficiency virus (HIV) utilizes CD4 as a receptor and CCR5 and/or CXCR4 as coreceptor to gain entry into the cell. The CCR5-tropic viruses, observed early in infection, could be important in transmission and the CXCR4-tropic viruses, observed late, may play an important role in disease progression. Viruses from 40 HIV-positive, asymptomatic or symptomatic individuals in India were isolated. Of 40 isolates 39 used CCR5. Thirty-three isolates were subtype C, 3 isolates were subtype A, and 4 isolates were HIV-2. Only 1 HIV-2 isolate, from a symptomatic individual, was dualtropic. Therefore, a majority of isolates from India belonged to subtype C and all the isolates utilized CCR5 exclusively irrespective of HIV disease status.

Introduction. The human immunodeficiency virus type 1 (HIV-1) uses chemokine receptor CCR5 and/or CXCR4 as the major coreceptor along with CD4 to gain entry into cells. Coreceptor usage came into prominence with the discovery of refractoriness to HIV infection in individuals homozygous for the δ-32 mutation in the CCR5 gene (*1, 2*). Coreceptor usage has been shown to correlate with the syncytium-inducing phenotype of the virus. The non-syncytium-inducing viruses use CCR5 while the syncytium-inducing (SI) viruses use multiple coreceptors, either one or several of the other coreceptors, i.e., CXCR4, CCR3, CCR2B, CCR8, GPR15 (BOB), STRL33 (Bonzo), and CX_3CR1, in addition to CCR5 (*3*). HIV-2 isolates have also been shown to be promiscuous in their coreceptor usage (*4*). Several studies have shown that virus isolates from HIV-1-positive individuals early in infection are non-syncytium inducing or CCR5-tropic (R5 viruses) while isolates obtained late in the course of disease are syncytium-inducing or CCR5/CXCR4-tropic (R5/X4 viruses) (*5*). Isolates from AIDS patients were also reported to use CXCR4, CCR3, CCR2B, CCR4, and BOB in addition to CCR5 (*6*). Several reports have thus shown an association between disease progression and switch in coreceptor usage. In an *ex vivo* study, CXCR4 specificity was shown to be a causal factor in $CD4^+$ T cell depletion, supporting the hypothesis that usage of CXCR4 accelerates immunodeficiency (*7*).

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In this study 40 HIV strains isolated from HIV-infected individuals in India were tested for their coreceptor usage. The individuals were classified as symptomatic or asymptomatic based on their clinical presentation. The $CD4^+$ T cell counts were also determined in 20 blood samples. The subtype of all the isolates was determined by the heteroduplex mobility assay (HMA). The study was undertaken to assess whether the coreceptor usage of viruses isolated from patients early in the course of infection differs from that of viruses isolated from patients who had various opportunistic infections indicating development of AIDS.

Results. Profile of HIV-positive individuals. A total of 40 primary isolates were obtained from blood collected from HIV-positive individuals residing in different parts of India. The majority of the isolates were from western India—Maharashtra ($n = 25$), Goa ($n = 6$), Gujarat ($n = 2$) and others were from South India ($n = 3$), North India ($n = 3$), and East India ($n = 1$). The mode of transmission was largely heterosexual. Of the 40 cases, only 3 had acquired the infection through blood transfusion and 1 by vertical transmission. Of the 40 HIV-positive individuals, 37 were in the age range of 22–47 with a median of 32. Of the remaining 3, 2 were above the range, ages 52 and 63, and 1 was 6 years of age.

Classification of HIV-Positive Individuals. The absolute $CD4^+$ T cell count was determined for 20/40 individuals, who were classified according to the CDC AIDS surveillance case definition—1993 (*8*) (Table 1). Of the 20 patients, 7 were in the early stage of HIV infection, while the remaining 13 were in the advanced stage of HIV disease

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TABLE 1

HIV Disease Staging (CDC AIDS Surveillance Case Definition, 1993) and $CD4^+$ T Cell Counts of Patients

CDC AIDS disease case stage	*n*	$CD4^+$ T cell count (cells/mm^3)				
		<50	50–100	100–200	200–500	>500
Early stage						
A1	1	Nil	Nil	Nil	Nil	1
A2	2	Nil	Nil	Nil	2	Nil
B1	2	Nil	Nil	Nil	Nil	2
B2	2	Nil	Nil	Nil	2	Nil
Late stage (AIDS)						
A3	2	Nil	Nil	2	Nil	Nil
B3	4	2	1	1	Nil	Nil
C3	7	3	3	1	Nil	Nil

or AIDS. Of those in the advanced HIV disease stage, 5 had $CD4^+$ T cell counts of fewer than 50 cells/mm^3, 4 had 50–100 cells/mm^3, while 4 had between 100 and 200 cells/mm^3.

Of the patients in whom $CD4^+$ T cell counts were not done, 6 were asymptomatic and 1 had an infection not particularly related to AIDS. Of the remaining 13, 4 had pulmonary tuberculosis, 4 had tubercular lymphadenitis, 1 had tubercular cold abscess, and 4 had HIV-related conditions.

Subtyping of Isolates by HMA. Four of the 40 isolates were HIV-2 by serological tests (*9*). The 36 HIV-1 isolates were subtyped using HMA (*10*) (Table 2). Three isolates belonged to subtype A of which 2 were genotyped as A3 (prototype Rwanda) and 1 as A1 (prototype Rwanda). The rest of the 33 HIV-1 isolates belonged to subtype C. On further genotyping, 25 had closest homology to C3 (prototype India/South Africa), 4 were determined to be C2 (prototype Zambia), and 1 was determined to be C1 (prototype Malawi) genotype. Thus a majority of HIV-1 isolates belonged to subtype C (33/36).

Coreceptor Usage of Isolates. The coreceptor usage of the HIV-1/2 isolates was determined using the infectivity assay in GHOST cells expressing either CXCR4 or CCR5 (*11*). The mean number of fluorescent cells in uninfected GHOST–CXCR4 was 16 ± 12 and in GHOST–CCR5 cells, it was 13 ± 8, based on 12 observations each. The mean value obtained in uninfected cultures + 3 SD was considered the negative cut-off. Thus values of more than 50 fluorescent cells/15–20,000 events were considered positive for virus infectivity. Figure 1 shows the representative scattergrams of GHOST–CXCR4 and GHOST–CCR5 cells infected with dual-, CXCR4-, and CCR5-tropic viruses. The laboratory-adapted strains of HIV-1, RF, IIIB, and MN were used to standardize the infectivity assay in GHOST cells. While the RF and IIIB strains infected only GHOST–CXCR4 cells, the MN strain showed 1% infectivity in GHOST–CCR5 cells, against 24.5% in GHOST–CXCR4 cells. The percentage of infected cells with primary isolates varied from 0.8 to 65%.

Of the symptomatic individuals, 32/33 virus isolates used CCR5 exclusively (Table 2). Twenty of the 33 symp-

TABLE 2

Subtype and Coreceptor Usage of the HIV Isolates

Symptomatic			Asymptomatic		
Subtype (genotype)	No. of patients	Coreceptor use	Genotype	No. of patients	Coreceptor use
HIV-1					
A (A1)	1	CCR5	A (A3)	1	CCR5
(A3)	1	CCR5	C (C3)	4	CCR5
C (C1)	1	CCR5			
(C2)	4	CCR5			
(C3)	21	CCR5			
(ND[a])	3	CCR5			
HIV-2	1	CCR5	HIV-2	2	CCR5
	1	CCR5/CXCR4			

[a] ND—not done.

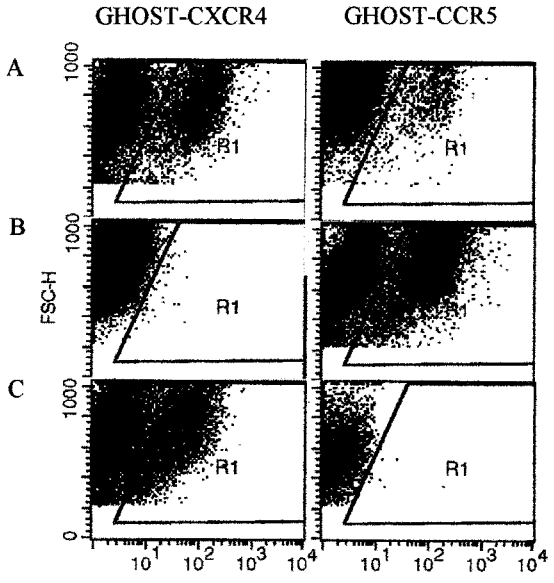

FIG. 1. Scattergrams derived from the FACScan analysis showing GHOST–CXCR4 and GHOST–CCR5 cells infected with different HIV-1 isolates. The region R1 delineates the fluorescence-positive infected cells. (A) The dualtropic HIV-2 primary isolate, (B) a CCR5-tropic HIV-1 primary isolate, and (C) the CXCR4-tropic laboratory-adapted RF strain.

tomatic cases had $CD4^+$ T cell counts done. Of these 13 had counts of <200 cells/mm^3 and thus would have been expected to have CXCR4-tropic viruses. Of these, 6 individuals had tuberculosis infections. The only dualtropic isolate was from an HIV-2-infected individual. The HIV-2 status of this individual was based on immunoblot results. This was confirmed by PCR amplification of the *env* gene using HIV-2-specific primers (*9*). The isolate showed 50% infectivity on GHOST–CXCR4 cells versus 7.3% on GHOST–CCR5 cells. The virus isolates from the 7 asymptomatic individuals were all CCR5-tropic.

Discussion. India, compared to any other country, is projected to have the largest burden of HIV infections. In a study of the isolates from Pune, India, 96% of the isolates were subtype C of which 66% were designated C3 genotype with maximum homology to the Indian C3 reference strain (*10*). In the present study 91.7% of the HIV-1 isolates were subtype C. Subtype C is also predominant in most recent HIV-1 epidemics worldwide (*12*). Recently it was reported that patients infected with subtype C isolates developed AIDS earlier than patients infected with subtype A virus (*13*). The presence of three or four NF-κB sites in subtype C viruses is thought to improve viral transcription (*14*) and thereby increase the pathogenicity of the virus.

Several studies have shown that broadening of the coreceptor usage profile of HIV-1 isolates may be associated with progression of disease to AIDS; 50% of the isolates from AIDS cases showed multiple coreceptor usage (*3*). A study using sequential isolates from four rapid progressors, six late progressors, and three long-term nonprogressors showed that the switch from CCR5 usage to multiple coreceptor usage occurred in all four rapid progressors and three of six late progressors (*6*). The emergence of multiple coreceptor-utilizing variants preceded CD4 depletion to <200 million/L and correlated with development of AIDS.

In the present study coreceptor usage of isolates from asymptomatic and symptomatic HIV-infected individuals from India was determined. HIV-infected individuals with tuberculosis were also classified as AIDS cases as per the CDC AIDS case definition. Persons having a disorder indicative of the progression toward AIDS were treated as symptomatic.

With the 33 subtype C viruses analyzed in this study, we observed no difference in the coreceptor usage of isolates from HIV-positive individuals at both ends of the disease spectrum, with $CD4^+$ T cell counts as low as 23 or as high as 905. Analysis of the V3 sequence of Indian isolates reported so far (*15–17*) revealed that the serine at position 11, which is essential for CCR5 usage, was present in all the strains and two arginine residues at positions 8, 11, and/or 18 needed for CXCR4 usage (*18*) were absent in all strains. The predominance of CCR5-using isolates has been recently reported for isolates from Ethiopia. A low frequency (6%) of SI viruses using CXCR4 and/or CCR5 and/or CCR3 was observed in 48 Ethiopian AIDS patients by Abebe *et al.* (*19*). In another study from Ethiopia, Bjorndal *et al.* (*20*) studied coreceptor usage in 9 AIDS patients. All isolates were classified as subtype C and all were found to use CCR5 as the coreceptor. A lower representation of CXCR4 usage was also reported in subtype C isolates from Malawi (*21*). In a study using biological clones of HIV-1 isolated both early and late from progressors and long-term survivors with wild-type or mutant CCR5/CCR2b genotypes, all the clones were restricted to the use of CCR5 (*22*), thus leading to the conclusion that an expanded coreceptor repertoire is not a prerequisite for progression in the clinical course of disease.

There have been several recent reports on the significance of CCR5 in viral pathogenesis *in vivo.* The natural knockout δ-32 mutation in CCR5 was shown to confer resistance to infection (*1, 2*). The promoter allele of CCR5 P1 was shown to be associated with rapid development of AIDS. Genetic association analysis of five cohorts of people with AIDS revealed that 10–17% of patients who developed AIDS within $3\frac{1}{2}$ years of HIV infection were homozygous for CCR5P1/P1 (*23*). Isolates from cases of dementia were shown to infect microglia, using predominantly CCR5 (*24*). Subtype C viruses predominantly occur in developing countries, where HIV-infected individuals are exposed to a larger load of communicable infections. Up regulation of CCR5 on macrophages infected with *Mycobacterium tuberculosis* has been reported (*25*). In human-PBL-SCID mice R5 strains were more pathogenic when the human cells exhibited

marked activation, 2 weeks after hu-PBL transfer (*26*). It has been reported that activation of circulating T lymphocytes down regulates expression of CXCR4 and thus interferes with propagation of X4 HIV strains (*27*). The superantigen, staphylococcal enterotoxin A, induced a marked decrease in expression of CXCR4 in activated cells positive for CD25. This could be a contributing factor in developing countries to the nonemergence of X4 viruses or it could be a characteristic of solely subtype C viruses.

This is the first report from India on coreceptor utilization by HIV isolates obtained from symptomatic and asymptomatic individuals, establishing an exclusive prevalence of CCR5-tropic viruses in the late stage of HIV-1 infections in the subcontinent. The findings thus assume importance in HIV management especially in the light of a recent report. Analysis of the evolution of virus diversity in HIV disease led to the conclusion that initiation of HAART before the transient appearance of X4 isolates may be more beneficial (*28*). Therefore, further studies need to be undertaken to study the evolution of HIV diversity in subtype C virus infections in the subcontinent and its implications.

Materials and Methods. Virus isolates. A total of 40 primary HIV-1 or HIV-2 isolates were used in the study. The isolates were from patients who resided in different parts of India. HIV-1 strains MN, IIIB, and RF were obtained from the Johns Hopkins School of Medicine.

Cells. Peripheral blood mononuclear cells (PBMCs) and SupT1 cells were cultured in RPMI 1640 with 20 and 10% FBS, respectively. The PHA-P-stimulated PBMC cultures were supplemented with 5 IU/ml interleukin-2. GHOST-clone 3 cells expressing either CCR5 or CXCR4 were obtained from Dan Littman (New York University Medical Center). The GHOST cells are human osteosarcoma cells genetically manipulated to express CD4 and one of the coreceptors with green fluorescent protein (GFP) as the reporter gene driven by the HIV-2 promoter. The GHOST cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, Geneticin (200 μg/ml), hygromycin (25 μg/ml), and puromycin (1 μg/ml). The cultures were maintained at 37°C in a 5% CO_2-containing, humidified incubator.

Collection of Sample. Samples were collected from individuals after obtaining their consent and anonymity was maintained throughout. Blood was collected by venipuncture into Vacutainers containing EDTA. The specimens were transported to NARI, Pune, and PBMCs were isolated within 48 h. The relevant demographic and clinical data were obtained at the time of sample collection.

Determination of CD4 Count. An aliquot of the blood sample was directly used for determining the lymphocyte profile. The absolute $CD4^+$ T cell count was estimated on the FACSort (Becton–Dickenson) with the Simulset software using monoclonal antibody panel consisting of anti-CD45/CD14, anti-1/2 anti-CD3, and anti-CD4 antibodies.

Determination of the HIV Disease Status. The CDC AIDS Surveillance Case Definition, 1993 (*8*), was used to stage HIV disease status in 20 cases for whom $CD4^+$ T cell counts were determined. In the remaining 20 cases the HIV-infected individuals were assessed for any HIV-related disorder and then classified as symptomatic or asymptomatic.

Virus Isolation. Viruses were isolated as described by Kulkarni *et al.* (*9*). Blood was collected from HIV-seropositive individuals and the PBMCs were separated on Ficoll–Hypaque. The patient's lymphocytes were cocultured with PHA-P-stimulated lymphocytes from HIV-negative donors in the presence of interleukin-2 (5 IU/ml) for 28 days. Fresh PHA-P-activated, heterologous, lymphocytes were added to the culture every week. Culture supernatants were collected at intervals of 3–4 days and stored at −70°C. Presence of HIV-1 p24 antigen, determined by ELISA (Organon Teknika, NL), was considered an indicator of virus replication.

Determination of the Subtype by HMA. HIV-1 subtyping was carried out as described by Gadkari *et al.* (*10*). Briefly, DNA was extracted from PBMCs using either the Isoquick DNA extraction kit (Orca Research Laboratories, U.S.A.) or the QIAamp DNA purification kit (Qiagen, U.S.A.). A region of the HIV-1 envelope (*env*) gene, including V3, V4, and V5, was amplified using two rounds of PCR. A 5-μl sample from the nested PCR product of each sample was mixed with 5 μl of individual plasmid PCR products representing reference strains of subtypes A1, A3, B2, C1, C2, C3, and E2 (obtained from the NIH AIDS Research and Reference Reagent Program). To each sample 1.1 μl of HMA buffer (100 mM NaCl, 10 mM Tris, pH 7.2, 2 mM EDTA) was added, and the sample was denatured at 94°C for 2 min and reannealed by quick chilling on ice. The resultant homo- and heteroduplexes formed between sample and reference strains were separated by polyacrylamide (5%) gel electrophoresis under nondenaturing conditions at 250 V for 3 h. The gels were then stained with ethidium bromide for 1 h.

The homoduplexes showed the fastest mobility. The sample/reference heteroduplex which migrated closest to the corresponding homoduplex determined the subtype designation. Some of the samples were further analyzed to determine the genotype within the subtype.

Infectivity Assay to Determine Coreceptor Usage. The method followed was as described by Cecilia *et al.* (*11*). Briefly, 70% confluent monolayers of GHOST cells expressing either CCR5 or CXCR4 were infected with virus stocks diluted 1:2 in the presence of 8 μg/ml DEAE-dextran (Sigma). Virus was allowed to adsorb overnight. Residual virus was removed; the cell sheet was washed and fresh medium containing 10% FBS

was added. Cells were harvested on day 3 or 4 postinfection, the day of virus addition being considered day 0. The infected cells were resuspended using 1 mM EDTA and fixed with 2% formaldehyde. The cells were then analyzed with the FACSort flow cytometer. The live cells were gated on the basis of forward and side scatter. The number of infected cells was determined by using a scattergram of fluorescence versus forward scatter after setting the gates with uninfected cells (Fig. 1). A total of 15–20,000 cells were scored. The mean number of fluorescent cells in the uninfected cell cultures + 3 SD was considered the cut-off value and cultures with values above the cut-off were considered positive for virus growth.

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